MEETING

ABSTRACTS OF PRESENTATIONS ON PLANT PROTECTION ISSUES AT

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Part 3 (Final Part)*

Characterization of Genes of a Bipartite Geminivirus Associated with Acquisition and Transmission by the Whitefly *Bemisia tabaci*

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African cassava mosaic virus (ACMV) and other bipartite geminiviruses have been used to study the molecular basis of transmission by the whitefly vector, *Bemisia tabaci*. Mutants and pseudorecombinants of insect-transmissible and insect non-transmissible clones of one Kenyan and two Nigerian isolates were used to study the role of coat protein (CP) and BCI movement protein. Insect specificity and transmission are controlled by the CP, whereas acquisition is a complex interaction of movement protein, feeding behavior and environmental factors. Insect-transmissible and non-transmissible clones had different symptom phenotypes Host factors may also contribute to the non-acquisition by whiteflies *(L)*

Multiplication and Transovarial Transmission of Tomato Yellow Leaf Curl Virus (TYLCV) in its Vector, the Whitefly *Bemisia tabaci*

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TYLCV is a geminivirus transmitted by the whitefly *Bemisia tabaci*. Several of the features associated with geminiviral replication in plants were found also in *B. tabaci* Following 1 h access to infected tomato, TYLCV DNA and proteins associated with whiteflies were analyzed during feeding with sucrose through membranes. TYLCV genomic DNA accumulated in the insects; the viral DNA was radiolabeled when [α-^{32}P]dCTP was added to the sucrose solution The TYLCV capsid protein started to accumulate at approximately the same time as did the viral genomic DNA The DNA strand complementary to the virus genome and the Rep protein were detected immediately after the initial access to infected plants TYLCV invades many tissues of the insect including the reproductive system It was detected in maturing eggs by *in situ* hybridization. Progeny (eggs, crawlers, adults) of insects that fed on infected tomato and were raised on TYLCV non-host plants contained large amounts of viral DNA Approximately 8% of the adults issued from viruliferous insects were able to inoculate tomato test plants *(L)*

Molecular Determinants Governing Beet Western Yellows Virus (BWYV) Transmission by Aphids

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BWYV is transmitted in a circulative, non-propagative manner by aphids Virions contain a minor protein, P74, produced by translation extension of the coat protein (CP) cistron into the neighboring readthrough domain (RTD). The RTD contains determinants essential for BWYV acquisition/transmission by *Myzus persicae*. Subdomains within the RTD are being functionally characterized by site-directed mutagenesis followed by aphid transmission assays. Inoculum was delivered to aphids as purified virus, leaves of agro-infected plants or extracts of transcript-infected protoplasts In-frame deletions in the C-terminal, variable half of the RTD generally did not abolish acquisition/transmission but greatly reduced its efficiency By contrast, the N-terminal, conserved half of the RTD appears to be largely essential for acquisition/transmission. Deletion of the proline hinge between the CP and the RTD abolished incorporation of P74 into virions and no acquisition/transmission was observed The effect of RTD mutations on virus accumulation in agro-infected plants was also discussed *(L)*

Partial Characterization of a Potyvirus Isolated from *Vernonia amygdalina*

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A potyvirus inducing mosaic and green vein-banding symptoms was isolated from bitterleaf (*Vernonia amygdalina*) from Lagos, Nigeria The virus was readily transmitted by mechanical inoculation. It had a narrow host range restricted to *Nicotiana benthamiana, Chenopodium quinoa* and *C. amaranticolor* It was also transmitted by *Myzus persicae* in a non-persistent manner The virus was purified from *N benthamiana*, and flexuous rod-shaped particles of ~750 nm were observed in purified preparations and leaf dips of *Vernonia* sp. Pinwheels and scrolls were observed in ultrathin sections of bitterleaf by electron microscopy The molecular mass of the coat protein was 34 kDa The virus reacted weakly with antisera to cowpea aphid-borne mosaic potyvirus (CAMV-Mor) and bean common mosaic potyvirus (BCMV-NY15) in dot-blot immunoassay. These results confirm that the hitherto difficult-to-transmit Vernonia green vein-banding virus is a member of the potyvirus group. *(P)*

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L = lecture sessions; *P* = poster (market place) sessions.

Comparison of the Helper Component (HC) Genes of Aphid Non-transmissible and Transmissible Spanish Plum Pox Virus (PPV) Isolates

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PPV is an aphid-transmitted potyvirus which causes serious diseases in plum, peach and apricot Two Spanish PPV isolates, PPV 5 15 and PPV 3.3, have been characterized as aphid-transmissible and non-transmissible, respectively, in plant-to-plant transmission tests by the aphid *Myzus persicae*. The isolates represent serologically distinct coat proteins of different molecular masses Since both isolates are aphid-transmitted when acquired through artificial membranes from purified virus preparations supplemented with purified HC obtained from potato virus Y-infected plants, a comparative analysis of their HC genes was performed. The results indicate that single amino acid changes also differentiate the HC of PPV 5.15 from that of PPV 3 3, which could be responsible for the lack of transmissibility of the PPV 3.3 isolate. [Research supported by grant PB 94-0023 from CICYT] *(P)*

Discrimination of Soybean Dwarf Luteovirus (SDV) Strains by Using Monoclonal Antibodies

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Two strains of SDV – SDV-YS and SDV-YP – are transmitted specifically by the aphid vector *Aulacorthum solani* and by *Acyrthosiphon pisum*, respectively They cannot be distinguished by host range tests or double antibody sandwich ELISA using polyclonal antisera Among the monoclonal antibodies (MAbs) raised against SDV-YS and SDV-YP, two reacted only with the former, and the other two only with the latter in triple antibody sandwich ELISA The reactions of these MAbs to ten field isolates was consistent with their vector specificity, demonstrating the validity of the MAbs for diagnosis of the two strains A survey of field isolates using these MAbs revealed that each strain is geographically localized *(P)*

Location of Domains with Specific Function at the N-terminal Region of Maize Dwarf Mosaic Potyvirus (MDMV)

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Aphid transmission of MDMV is dependent on the intact coat protein (CP) on the one hand and the presence of a viral coded helper component (HC-pro), on the other. Proteolytic removal of the coat termini abolishes aphid transmission Recently we demonstrated that the N-terminal CP region has the potential to compete with the intact virions on aphid transmission. This region of MDMV, ~60 amino acids long, was cloned and expressed in bacteria Polyclonal MDMV antibodies recognize the bacterial expressed polypeptide. When the DAG domain located in five amino acids from the amino terminus was deleted, the bacterial expressed polypeptide was still recognized by the antibodies, although it lost its ability to inhibit aphid transmission. To map the antigenic domain that is located in the N-terminal CP, constructs each half the size of the N-terminal region were prepared, cloned and expressed in bacteria. The effect of these small polypeptides on aphid transmission, antibody recognition was examined. The potential interaction with the HC-pro is under investigation The possibility to transform plants with such minigenes, in order to limit virus spread, was discussed. *(L)*

Mutations in the HC-pro Gene of the Zucchini Yellow Mosaic Virus (ZYMV) and Their Effect on Transmission by Aphids

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Two conserved regions were associated with the helper protease (HC-pro) activity in assisting aphid transmission of potyviruses (the KITC region and the PTK region). The possible function of the PTK region was investigated by replacing the threonine by amino acids of a different polarity (Thr to Val) or size (Thr to Ser), using the infectious full length clone of ZYMV. Plants were inoculated with the new HC-pro mutants (ZYMV-PVK and ZYMV-PSK) as well as with the wild-type (ZYMV-PTK) or a mutation occurring in a wild-type helper-deficient HC (ZYMV-PAK). The HC-pro mutants were compared for helper activity in aphid transmission and for symptoms severity. The HC extracted from either ZYMV-PVK or ZYMV-PSK was capable of assisting in transmission of purified ZYMV virions, but at lower efficiency than the wild type. The titer of virus was determined for each of the four mutants by ELISA and the level of HCs was visualized by Western blots The possible role of the PTK region in aphid transmission was discussed. *(L)*

Context of the Coat Protein DAG Motif Affects Potyvirus Transmissibility by Aphids

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Full-length clones derived from two potyviruses (tobacco vein mottling virus, TVMV, and tobacco etch virus, TEV) have been used to introduce specific changes in their coat proteins (CP) by site-directed mutagenesis and the creation of new restriction sites by PCR Mutations were designed to define the context in which the conserved motif *asp-ala-gly* (DAG) retains activity for aphid transmission The sequence of the CP N-terminal region of the highly aphid-transmissible isolate of TEV contains two consecutive DAG motifs separated by a single *ala*. In this original context, transmissibility was abolished or strongly diminished by mutations affecting the first or both DAG motifs, while mutations of only the second motif had little or no effect. In certain contexts, however, the second DAG could be made functional for transmission by substitution of *val* for *ala* in the position immediately before the second DAG Conversely, changing the *val* located prior to the first DAG to *ala* resulted in reduced transmissibility In contrast, mutations of the *val* located before the single DAG motif of TVMV did not affect transmissibility, nor did the creation of a second DAG motif at the beginning of the TVMV CP core restore transmissibility of a DAE mutant. All mutants infected tobacco plants with the exception of those with substitutions of *lys* or *arg* for *asp* in the first position of the DAG motif in TVMV, or the double substitution of *lys* for *asp* in the two DAG motifs in TEV These mutations seemed to affect negatively virus movement in the plant, since both TVMV mutants were able to infect tobacco protoplasts *(L)*

Genetic Determinants Involved in the Transmission of Tobacco Rattle Virus (TRV) by *Paratrichodorus pachydermus*

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TRV is the type member of the tobravirus group. Its genome is bipartite and consists of RNAs 1 and 2. RNA 1 encodes the viral replicase and movement functions, RNA 2 encodes the coat protein (CP) and, depending on the strain, several non-structural proteins. Transmissibility of TRV is determined by RNA 2, where the CP is not the only factor involved. TRV isolate PPK20 is transmissible by *Paratrichodorus pachydermus* and sequence analysis shows the presence of two putative non-structural proteins of 29 4 and 32 8 kDa, respectively Transmission studies of deletion mutants of TRV-PPK20 revealed that the 32 8 gene was dispensable and that the 29 4 gene was required for transmission by *P pachydermus (L)*

Molecular Epidemiology of Tomato Spotted Wilt Tospovirus (TSWV)

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TSWV, a member of the genus *Tospovirus* (family Bunyaviridae), has become a major constraint to the cultivation of peanut and tobacco in Georgia A multidisciplinary approach is being pursued to understanding the virus biology, molecular biology, epidemiology and host resistance, with a view to developing a disease management program Information on the various strains of the virus that are prevalent in Georgia, and the ability to determine the proportion of viruliferous thrips and the source(s) of primary inoculum, will facilitate a better understanding of the basis for the epidemics in Georgia The nucleocapsid protein genes (N gene) of TSWV isolates collected from various parts of the state are isolated by RT-PCR and typed either by restriction analysis or nucleotide sequencing Sequences unique to the Georgia isolates have been identified Thrips species known to transmit TSWV in Georgia (*Frankliniella occidentalis* and *F fusca*) are collected during the non-crop period (winter) as well as the growing season, and the proportion of viruliferous thrips is determined by ELISA using a monoclonal antibody specific to the non-structural gene product (NSs) of TSWV. These approaches should provide the genetic identity of the various isolates of TSWV infecting peanut, tobacco and vegetables in Georgia. *(P)*

A Single Amino Acid Change in the Cucumber Necrosis Virus (CNV) Shell Domain Significantly Reduces Vector Transmission

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CNV is transmitted *in vitro* by zoospores of the fungus *Olpidium bornovanus*. CNV coat protein (CP) is the determinant of the specificity of transmission. CNV variants deficient in fungus-transmissibility were identified by testing virus from individual local lesions of mechanically passaged CNV for fungus transmission Virus from one local lesion (designated LL5) showed significantly reduced transmission Analysis of this mutant showed that it contained two aa substitutions one in the arm (Phe to Cys) and the other in the shell domain (Glu to Lys) These mutations were analyzed in separate infectious clones and it was found that the Glu to Lys change in the shell is responsible for the reduction in transmissibility The Glu to Lys change does not affect particle stability but is associated with altered particle mobility on agarose gels, suggesting that the loss of transmissibility is due either to an altered charge or a conformational change in the virus particle Studies are under way to determine if LL5 is able to bind zoospores *(L)*

Sequence Analysis of Leek Yellow Stripe Virus (LYSV) Isolates Differing in Biological and Serological Properties

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There are at least three serologically distinct members of the genus *Potyvirus* infecting *Allium* spp. Recent evidence suggests that there are host-adapted strains of LYSV, *e.g.* some isolates from leek and garlic do not infect garlic and leek, respectively. Moreover, our results from recent serological studies have shown that some of these host-adapted strains of LYSV can be clearly differentiated in DAS-ELISA and differ by up to four dilution steps in IEM decoration titer experiments. Since no nucleotide sequence data are available for LYSV, these findings prompted us to study the molecular basis for the striking serological differences observed among three LYSV isolates from garlic and leek a German isolate from leek (LYSV-L), a Taiwanese isolate from garlic (LYSV-G) and an Indonesian isolate from leek (LYSV-510) Nucleotide sequence analysis of the 3' end of the genomic RNA of these isolates showed that they have nearly identical coat protein (CP) sizes and their CP amino acid sequences have identities of 83-95% Moreover, their 3'-NTR regions share identities ranging from 89-96% These data suggest that all isolates studied are strains of LYSV and support the concept that some biologically and serologically distinct LYSV strains have evolved as a result of the exclusively vegetative propagation of certain *Allium* species in geographic isolation. *(P)*

Detection of Virus Particle-helper Component Complexes in *Nicotiana benthamiana* Plants Infected with Plum Pox Virus (PPV)

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The interaction of virus particles and helper component (HC) protein in extracts from plants infected with PPV was examined For this purpose, a sensitive method of detection of virus particles after native electrophoresis was developed. Sap from different *Nicotiana benthamiana* plants was electrophoresed under natural conditions, and blotted to nitrocellulose membranes

Polyclonal antisera, specific for the coat protein (CP) and the HC, obtained after immunization with purified recombinant CP and HC proteins, were used to localize the position where free CP and HC proteins, virus particles, or complexes of both virus particles and HC migrated Free CP and HC subunits migrated much faster, being easily distinguishable as a smear at greater distances The presence of virus particles was detected at shorter distances in the gel by Western blot, and identified by IEM. In several experiments with extracts from different tissues, we were able to detect HC and virus particles at the same level, indicating that the two entities moved as a complex. Such complexes were detected in leaves, midribs, stems and roots of the infected plants tested. *(P)*

Aphid Transmission of Potato Spindle Tuber Viroid (PSTVd) Encapsidated by Potato Leafroll Luteovirus (PLRV) Particles

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Myzus persicae aphids were able to transmit PSTVd to healthy *Physalis floridana* from *P floridana* plants co-infected with PLRV, whereas they did not transmit the viroid from plants infected with PSTVd alone. Dot-blot hybridization was used to detect PSTVd. In the aphid-inoculated *P. floridana*, PSTVd was detected only in plants that also became infected with PLRV Doubly infected plants were used as sources of inoculum for subsequent aphid transmissions to the healthy tomato cv 'Rutgers', *P floridana* and *Datura stramonium* PSTVd was detected by dot-blot in 17 of 30 plants of tomato In *P. floridana* and *D stramonium*, the viroid was not detected by dot-blotting, but it was detected in some plants by the recovery test on Rutgers plants. The results indicate that transmission of PSTVd by *M persicae* was assisted by PLRV Possible *in vivo* encapsidation of PSTVd by PLRV particles has been proved in the experiment with treatment of highly purified PLRV preparations with RNase *(L)*

XIX: ENCAPSIDATION

Host Effect on Accumulation and Encapsidation of Defective-Interfering RNAs in Broad Bean Mottle Virus (BBMV) Infections

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BBMV encapsidates defective-interfering (DI) components that are formed by in-frame deletions within the RNA 2 open-reading frame. The biological characteristics of these DI RNAs include exacerbation of the severity of symptoms on certain hosts and the lack of DI RNA through other hosts In order to study the molecular aspects of the relationship between DI RNA transmission and symptoms, we investigated the DI RNA accumulation and encapsidation in various local lesion or systemic host plants This was analyzed using Northern blots and RT-PCR of total and virion RNA preparations Whereas many systemic hosts transmitted and encapsidated efficiently, the DI RNAs during BBMV infection of bean or pea plants did not Similarly, local lesion hosts did not accumulate or encapsidate DI RNAs The nature of the observed effects on pea and bean hosts was analyzed further by studying the accumulation of DI RNAs in the corresponding mesophyll protoplasts *(P)*

Effects of Cucumber Mosaic Virus (CMV) Capsid Protein Deletions on RNA Binding and Assembly

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Deletions were engineered into the capsid protein (CP) gene of CMV, resulting in deletions of amino acids 26-40 (δSac) or 15-40 (δSal) in the corresponding viruses. The δSal mutant showed restricted virus movement in tobacco and squash vs the δSac mutant, which behaved like wild-type virus but with altered pathology Virions could not be purified from *Nicotiana clevelandii* systemically infected by either δSal or δSac mutants, and no virus particles could be detected by electron microscopic examination of infected tissue extracts. Western blot analysis showed the presence of truncated CP in δSal and δSac infected plants. These CPs could bind RNA *in vitro*; however, the δSal CP·RNA complex was much less stable than the δSac CP complex, which was only slightly less stable than the wild-type CP complex. These data suggest that the N-terminal region of the CMV CP is involved in the formation of virions *(L)*

Encapsidation of the Viral RNA of Turnip Crinkle Virus (TCV) is Directed Solely by a RNA Segment in the Coat Protein Gene

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TCV is a 30-nm icosahedral plant virus with a positive sense RNA genome of 4051 bases encapsidated by 180 copies of a 38-kDa capsid protein (CP) subunit An *in vivo* assay has been developed that permits reliable determination of viral RNA encapsidation in protoplast infections. Using this system we have been able to show that mutant viral RNAs unable to synthesize CP can be efficiently packaged *in trans* when co-inoculated into protoplasts with infectious RNA of wild-type TCV Analysis of the encapsidation of various mutant viral RNAs in protoplast infections has permitted the identification of 190-base region of the CP gene that is likely essential and sufficient for viral RNA packaging We are now delineating the limits of this RNA element by mutagenesis of the CP gene Furthermore, we have confirmed that the TCV CP gene itself is sufficient for assembly initiation *in vivo* by constructing a chimeric virus composed of the tomato bushy stunt virus (TBSV) genome but with the TBSV CP gene precisely replaced with the TCV CP gene. *(L)*

Transcapsidation in Transgenic Plants Expressing Potyvirus Coat Proteins (CP)

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Transgenic *Nicotiana benthiana* plants expressing the CP of either bean yellow mosaic virus (BYMV) or plum pox virus (PPV) were inoculated with different potyviruses in order to determine the potential risk of heterologous encapsidation of the transgenic CP in non-homologous virus particles. Virus preparations from infected plants were analyzed by ELISA, Western blotting, and immuno-specific electron microscopy (ISEM) Both transgene CPs were readily detected in purified heterologous virus particles, whereas the incorporation level of the transgene varies significantly depending on the transgenic plant line and on the virus that has been used for inoculation. ISEM with gold-labeled BYMV and PPV antibodies showed incorporation of BYMV CP into PPV particles and *vice versa* Further, ELISA data show in some transgenic plant lines increased detection of transgenic BYMV after PPV infection *(P)*

Encapsidated Deletion Mutants of Grapevine Fanleaf Virus (GFLV) RNA2

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Immunocapture reverse transcription polymerase chain reaction (IC/RT-PCR) from grapevine shoots naturally infected by GFLV was performed under touch-down conditions in order to amplify a 1698 nt sequence including the viral coat protein (CP) gene located at the 3' end of the polyadenylated GFLV RNA2 In addition to the anticipated product, the reaction repeatedly yielded high amounts of a shorter, unexpected cDNA fragment. Sequence analysis showed that it derived entirely from genomic RNA2 by a single internal deletion of 1367 nt within the CP gene In the following study, wild-type RNA2-specific primers and primers requiring deletion sites at RNA and cDNA level were used for reverse transcription and subsequent PCR, revealing two types of possible RNA2 deletion mutants with lengths of 403 nt and 464 nt, respectively, and polyadenylated 3' ends. Whereas the 3' extensions of these defective molecules derive from different regions of the GFLV genome, sequence alignment of their 3' extensions yielded 100% homology, showing that the 3' side of their deletion sites is identical. Since immunocapture technique was applied for particle isolation, it is likely that the potential RNA2 mutants described here are enveloped in viral CP and thus carry the signals required for packaging *(P)*

XX: VIRAL PROTEASES

Characterization and Mutagenesis of Tomato Ringspot Nepovirus (TomRSV) Protein Cleavage Sites

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TomRSV proteins are cleaved from precursor polyproteins by a virus-encoded serine-like protease It was shown previously that the TomRSV protease recognizes two cleavage sites on the polyprotein encoded by RNA-2 (P2) intermolecularly These cleavage sites contained a glutamine at the -1 position and a serine or glycine at the +1 position which are typical of como and potyvirus polyprotein cleavage sites but not of subgroup 1 nepovirus polyprotein cleavage sites. These cleavage sites also had a cysteine or valine in the -2 position and a serine in the -4 position which are also found in the P2 cleavage sites of at least one other nepovirus. These amino acids were also found on other predicted cleavage sites on TomRSV P1 and P2. A cleavage site between the protease and polymerase on P1 has recently been characterized *in vitro*. Identification of this and other cleavage sites on P1 and P2 is underway. A site-directed mutagenesis of amino acids at and around the N-terminal movement protein cleavage site was done. The results of the mutagenesis on cleavage site processing by the protease *in vitro* were shown. *(P)*

XXI: MOLECULAR TECHNIQUES OF DIAGNOSIS

Rapid and Large-Scale Screening of Carnation Etched Ring Virus (CERV) by Polymerase Chain Reaction

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Polymerase chain reaction (PCR) was used to identify CERV in leaves of infected carnations Two 20-mer oligonucleotide primers were designed to regions of the intergeneric sequence between VI. and VII genes. The 850 nt amplified fragment was cloned and sequenced, showing 95 6% identity with the published sequence. The cloned fragment was used after radioactive labeling to prove the viral origin of the PCR products amplified from the nucleic acid samples originated from plant sap Different DNA extraction methods were compared and a very simple one was chosen to screen large-scale samples In this survey up to 50% of the carnation samples were positive for the virus although most of the plants were symptomless. *(P)*

Diagnosis and Sequencing of Coat Protein Gene of Strawberry Vein Banding Virus (SVBV)

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SVBV was detected first by grafting in plants of the strawberry germ plasm collection as well as in strawberry plantations at several places in the Czech Republic during 1991-1995. A nonradioactive digoxigenine-labeled hybridization probe was prepared from the pSVBV-E3 clone obtained from the American Type Culture Collection. Strawberry samples exhibiting symptoms of SVBV-like infection gave positive reactions with this probe. Part of the SVBV genome containing the coat protein (CP) gene was cloned and sequenced. The deduced amino acid sequences showed only short, highly homologous regions in the C-terminal part of the gene with the other caulimoviruses. Primers suitable for amplification of the CP gene were synthesized and a methodology for routine PCR detection of the SVBV in strawberry plants was developed. *(P)*

Detection of Carmoviruses and Tombusviruses in Pelargonium Plants by Polymerase Chain Reaction (PCR)

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Pelargonium is one of the most important ornamental plants in Europe Since *Pelargonium* plants are propagated mainly by cuttings, control of viral and bacterial diseases depends essentially on the elimination of infected plants Improved programs for the production of certified pathogen-tested plants will be necessary and require the availability of suitable and sensitive indexing procedures, to ensure the production of the nuclear stock. In commercial cultures, several viruses infect *Pelargonium* plants, among them some specific viruses belonging to the Tombusviridae family, such as pelargonium flower-break carmovirus (PFBV), pelargonium leaf-curl tombusvirus (PLCV), pelargonium line pattern carmovirus (PLPV) and pelargonium ringspot carmovirus (PRSV). Immunoenzymatic methods (ELISA) are already available for PFBV and PLPV, but that detection is limited by an uneven distribution of the virus or a low virus titer in infected plants. We intend to improve the detection methods in crude extracts for the *Pelargonium* viruses using PCR procedures (RT-PCR and IC-RT-PCR). Synthetic primers were designed from conserved and non-conserved carmovirus sequences, derived from sequences already published and from sequences obtained from a PFBV cDNA library obtained in our laboratory. They were chosen for the simultaneous detection of tombusviruses (PLCV) and carmoviruses (PFBV, PLPV and PRSV), or the specific detection of PFBV, lately the most damaging virus on *Pelargonium*. *(P)*

Citrus Ringspot (CtRSV) and Citrus Psorosis-Associated Virus (CPsAV) can be Detected by RT-PCR and ELISA Assay

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Psorosis, responsible for important losses in the citrus industry worldwide, is probably caused by CPsAV Budwood certification, currently based on graft inoculation to indicator plants, is slow and expensive, but is the only procedure available to index for this unstable virus often present at low levels. It is thus important to develop a rapid and sensitive test for the virus. CPsAV 90-1-1 has been partially purified and characterized, and closely resembles CtRSV. The viruses have divided genomes, and the bottom component of CtRSV-4, when deproteinized, yields both ds and ssRNA We have cloned and sequenced sections of this dsRNA and used the sequences to design an RT-PCR reaction that detects both CtRSV-4 and CPsAV 90-1-1 from infected citrus plants. A new polyclonal antiserum to CtRSV-4 has also been produced, and a DAS-ELISA based on it also detects both viruses in citrus plants. *(P)*

The Use of Long-Distance Reverse Transcriptase-PCR (LD-RT-PCR) to Amplify Full Length Virus Genomes

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Full-length viral genome clones are an important tool for the study of viral replication mechanisms Until recently, most full-length viral genomes' clones were produced by arrangements of randomly cloned fragments. An alternative to this approach could be the use of long-distance PCR (LD-PCR) with appropriate primers A mix of thermostable enzymes, composed of Taq polymerase as the major component and a small quantity of proof-reading enzyme, made it possible to amplify DNA fragments of over 25 kbp Amplifying large full-length viral RNA genome by the LD-RT-PCR method has been problematic because, contrary to the ease with which LD-PCR is applied to DNA, it is difficult to synthesize large complementary DNA with the regular RT enzymes. We established the conditions for producing large cDNAs using various reverse transcriptases and then applied LD-PCR to obtain full-length viral genomes. Using the LD-RT-PCR that we developed, we amplified the 3.2 kbp A genome fragment of infectious bursal disease virus, the 6 3 kb genome of tobacco mosaic virus, and the 8.5 kb genome of foot-and-mouth disease virus *(L)*

Immunochemical and Biochemical Analysis of Some Potato Viruses by Means of Monoclonal Antibodies

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Monoclonal antibodies (MAbs) produced in our laboratory against various potato viruses were used for detailed characterization of immunochemical variability and antigenic properties of potato virus A, potato virus S (PVS) and potato virus Y Some selected hybridoma lines produced MAbs with narrow specificity (they were strain- or isolate-specific, *e.g.* PVS); on the contrary other hybridomas produced MAbs with sufficiently broad activity that they are suitable for large-scale utilization. *(P)*

Construction of a cRNA Probe Specific for the Detection of the Sour Cherry Strain of Plum Pox Virus (PPV-SoC)

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The nucleotide sequence of the N-terminal region of the coat protein (CP) gene and the C-terminal region of the NIb gene of PPV-SoC were determined in our laboratory and found to be significantly different from those of other known isolates of PPV A 447 bp DNA fragment containing 303 bp and 144 bp from the CP gene and NIb gene, respectively, was amplified from a cloned PPV-SoC genome and then subcloned into PCR II vector (Invitrogen). A non-radioactive DIG-labeled cRNA probe was generated from the subclone and used in dot blot hybridization assay with several PPV isolates including PPV-D, PPV-M, and PPV-El Amar. Additionally, a DIG-labeled cRNA probe derived from the conserved C-terminal region of the PPV CP gene was used for hybridization to ascertain that tested samples were infected with PPV. The PPV-SoC cRNA probe hybridized strongly to samples from PPV-SoC-infected tissues only The probe did not hybridize to samples infected with other isolates of PPV, which included PPV-D, PPV-M, and PPV-EL Amar. The PPV cRNA probe for the C-terminal region of the PPV CP gene hybridized strongly to all isolates of PPV, including those of PPV-SoC. Our results suggest that the PPV-SoC cRNA probe developed in this investigation may be used for studying the identity, epidemiology and geographical distribution of members of the PPV-Cherry (PPV-C) group *(P)*

Sequences Cloned from Sugarcane Striate Mosaic Disease (ScSMD)-Affected Plants Indicate Possible Virus Identity

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ScSMD is a disease of unknown etiology of limited distribution in northern Queensland A disease-associated dsRNA (*ca* 9kbp) has been isolated by cellulose chromatography. Reverse transcription of dsRNA with a random hexamer linked to a universal primer produced a cDNA library This library was then amplified by PCR primed with the universal primer (Froussard, 1992). The PCR products were cloned and selection was done by hybridization to the dsRNA. Four disease-specific clones were sequenced A database search revealed that the dsRNA had the greatest sequence homology with the carlaviruses in the putative replicase gene. This is the first evidence of a carla-like virus infecting sugarcane. Further work is in progress to isolate a putative virus particle and to develop a PCR-based diagnostic method for ScSMD. *(P)*

Genome Mapping: A Novel Method for Determining the Sequential Order of Restriction Digest Fragments of DNA

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A novel method is presented which enables the construction of restriction digest maps. The method involves reciprocal single and double digests of DNA using a pair of restriction endonucleases which have different cleavage target sequences.

Double digest products are aligned with their various parent single digest products in a second dimension gel electrophoresis separation Analysis of the origins of all double digest products leads to a complete linear (or where relevant circular) sequential order or map for all digest products. The efficacy of this method was tested using the bacteriophage λ double-stranded DNA genome whose complete base-pair sequence (48,502) is known and for which restriction sites can be predicted. The restriction endonucleases *BamH1* and *Sma1* were used in this study. Hybridization with radioisotope or other labeled probes is not a necessary part of this mapping procedure However, probes can be used to map specific regions, to provide confirmation or to resolve ambiguities. The procedure is also applicable in principle to any system for resolving DNA molecules and is particularly amenable to computer-aided mapping procedures. This method is applicable in principle to any double-stranded DNA molecule, whether linear or circular, whether a whole chromosome, whole genome or part thereof *(L)*

XXII· EXPRESSION OF THE GREEN FLUORESCENT PROTEIN (GFP) IN VIRUSES

Potato Virus X (PVX) as a Vector for Expression of Proteins in Plants

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Plants offer an attractive, alternate system for the production of proteins and peptides. Plant virus-based vectors have previously been used to express free proteins and peptides fused to viral coat proteins (CP). We have developed a vector based on PVX that produces a fusion between the GFP from *Aequorea victoria* and the CP of PVX The vector contains a sequence encoding the 2A peptide of foot-and-mouth disease virus, which disrupts peptide bond formation, between the genes for the GFP and the PVX CP The presence of 2A results in the production of a mixed pool of free CP and CP fused to GFP. The modified virus infects plants systemically and accumulates to high levels. Mutation of the 2A sequence shows that its processing is essential for virus assembly. The virus particles produced incorporate the GRP-CP fusion protein. The general utility of this vector for over-expression of proteins in plants and as an epitope presentation system is being tested *(P)*

Expression of the GFP Using Recombinant Baculovirus and Entomopoxvirus Vectors: Improved Selection of Recombinant Viruses

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Expression of the gene encoding GFP in insect cells using a recombinant *Autographa californica* nucleopolyhedrovirus (AcNPV) produces an intense green fluorescence that can be visualized under UV light in plaque-assays and in infected cells by light microscopy By incorporating *gfp, in lieu* of the polyhedrin gene, into the genome of an AcNPV modified to contain two unique *Bsu36I* sites, we have produced an efficient and reliable method for the production of recombinant viruses, it is an alternative to selection using the *E coli lacZ* gene. In the production of recombinant entomopoxviruses (EPVs), modified viruses are selected by their spheroidin-negative phenotype. Attempts to improve selection methods by utilizing *lacZ* or GUS reporter genes have failed, due largely to problems of host cell toxicity or impermeability to the chromogenic substrates employed. These problems should be overcome by the use of *gfp*, which has been introduced into the AmEPV genome *in lieu* of the spheroidin gene, permitting a simple screen for recombinant viruses at the plaque-assay stage. *(P)*

Expression of Green Fluorescence with Baculovirus Vector in Insect Cells

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The green fluorescence of bioluminescent jelly fish *Aequorea victoria* is due to the presence of the GFP. The ORF gene is 717 bp encoding 238 amino acid residues. To examine whether the GFP gene can be employed as a reporter gene in insect cells, a baculovirus transfer vector containing the neomycin-resistance gene (*neo*) was established. The GFP gene was subcloned into the vector downstream of the polyhedrin gene (*ocu*) promoter. The strong promoter of the *ocu* gene and weak promoter of the IE-1 gene account for the visible band of GFP and invisible band of *neo* on SDS-polyacrylamide gel. In the presence of G418, the recombinant virus can be purified as the *neo* gene expression Expression of the GFP gene in the recombinant virus should give rise to synthesis of the GFP with a mol.wt of 30 kDa, and is observable by the resulting strong green light irradiated by UV of blue light in viable, intact insect cells. The GFP produced in insect cells has typical fluorescent spectra indistinguishable from those of the purified native GFP The GFP gene as a good reporter gene can be used to monitor gene expression and protein localization in insect cells and other living organisms, it is more convenient than other reporter genes, having no requirement for an exogenous substrate and a co-factor for fluorescence. *(P)*

The GFP: A Visual Reporter and Fusion Partner for Insect Cells

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The gene encoding the GFP of the jellyfish, *Aequorea victoria*, was inserted under the transcriptional regulation of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus, AcNPV. The recombinant baculovirus was used to infect lepidopteran *Spodoptera frugiperda* and *Trichoplusia ni* insect cells. The fluorescence spectra of the insect-cell-expressed GFP was similar, if not identical, to that described for the authentic protein The recombinant protein, which migrated with an apparent molecular weight of 26 kDa, was easily detected, *in vivo*, by fluorometry at various time points after infection with the recombinant baculovirus. In fact, the features of this protein, in conjunction with a high level of expression, gave the infected insect cells a bright green color which was clearly visible in daylight In addition, a genetic fusion containing GFP and streptavidin of *Streptomyces avidinii*, was engineered and expressed in these insect cell lines The chimeric protein product retained a dual biological function, in that it was highly fluorescent and capable of binding biotinylated molecules. Nonreduced SDS-PAGE analysis, fluorometry, FACS analysis as well as fluorescence microscopy, revealed that GFP is a powerful reporter, or fusion partner, for baculovirus-infected lepidopteran insect cells *(P)*

XXIII· VIRUSES OF INSECTS AND OTHER INVERTEBRATES

Investigation of Baculovirus Gene Function Using Yeast Vectors

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Autographa californica nucleopolyhedrovirus (AcMNPV) DNA was maintained in *Saccharomyces cerevisiae* cells *via* the introduction of yeast-replicative elements (ARS, CEN and URA3) to derive yAcBK5. Virus DNA recovered from yeast cells was used to transfect *Spodoptera frugiperda* cells to demonstrate the production of infectious virus particles This system was then used to investigate whether or not the AcMNPV ORF1629, which is thought to encode a 78-kDa structural protein, was essential for virus replication ORF1629 was modified by inserting a second yeast-selectable marker, SUP4-o, into an Eco R1 site within the 3'-end of the coding region. The resulting plasmid, pUC1629-SUP4-o, was used to transform yAcBK5. Recombinant yeast cells were selected in the absence of adenine. Total DNA recovered from these cells was analyzed using PCR and hybridization techniques to establish that the SUP4-o coding region had been inserted within ORF1629. Subsequently, yAc1629-SUP4-o DNA was used to transfect insect cells, in the presence or absence of pUC1629 Only cells co-transfected with both DNA molecules yielded infectious virus, confirming that ORF1629 is required for virus replication. *(L)*

Possible Involvement of Insect Cells Nuclear Factor in the Regulation of the Delayed Expression of the Baculovirus Polyhedrin Gene

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The baculovirus expression system is widely utilized for the production of recombinant proteins in *Spodoptera frugiperda* Sf9 insect cells. The polyhedrin gene promoter (Ppolh) of the *Autographa californica* multiple nuclear polyhedrosis virus is used mainly to drive the expression of a protein of interest. However, the molecular mechanism that governs the very late expression of this promoter is still unclear. To understand this delayed regulation, we have used Electromobility Shift Assays with various segments of the Ppolh as well as mutated Ppolh. These segments were used to identify specific DNA binding factors that are found in nuclear extracts prepared from uninfected and infected cells at different time intervals Our results show that a host-encoded protein(s) binds specifically to the promoter region at positions -72 to -165; this binding activity was diminished as viral propagation progressed This DNA segment is characterized by multiple putative sites for GATA-like transcription factor Interestingly, this binding activity was noted also in nuclear extracts from *Spodoptera littoralis* cells that also diminished as viral infection progressed. The *S. littoralis* multiple nuclear polyhedrosis virus Ppolh also demonstrates the same DNA binding characteristic and also contains multiple GATA motifs. The data imply the presence of a host-encoded DNA-binding protein that acts as a negative regulator and is involved in the delayed expression of polyhedrin. Transient co-transfection assays were performed to assess the *in vivo* competition of proteins that interact with the Ppolh. The results support the concept of negative regulation Thus it is possible that baculovirus utilizes an insect-cell-derived nuclear factor to control the differential expression of late *vs* very late genes. *(L)*

Construction of a Versatile Recombinant Baculovirus Insecticide with a Multi-phenotype (ocu$^+$ /p10$^+$ /cryT$^+$ /neo$^+$ /PE$^-$)

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A polyhedral envelope (PE) gene-negative transfer vector pAc34DZ2 with the insertion of the neomycin gene was constructed Recombinant baculovirus vAcPhBtTPE$^-$ was obtained after *Spodoptera frugiperda* cells were co-transfected with previously constructed recombinant virus vAcPhBtT DNA mixed with pAc34DZ2 Southern blot indicated that the neomycin gene was integrated into the PE gene locus of the vAcPhBtTPE$^-$ genome. Microscopy indicated there were no differences between the recombinant virus and previously constructed vAcPhBtT in morphology, size or population Electron microscopy demonstrated that the recombinant virus lacked a polyhedral envelope. Alkali lysis of polyhedra showed that vAcPhBtTPE$^-$ released viron faster than vAcPhBtT. The vAcPhBtTPE$^-$ was a recombinant polyhedra-positive baculovirus with polyhedral envelope-negative, expression of truncated crylA (b) gene from *Bacillus thuringiensis*, and containing the neomycin gene as a screening marker. Bioassay and field spray showed that both recombinant baculoviruses were effective as insecticides, but vAcPhBtTPE$^-$ seemed to be more applicable for it could result in the dramatic reduction of the insect population, calculated 48 h after a field spray. *(L)*

Wiseana Iridescent Virus: Characterization of Temperature-Sensitive Replication

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Wiseana iridescent virus (WIV) is a member of the Iridoviridae family of viruses, a group of large (120–200 nm), icosahedral, dsDNA viruses that infect both vertebrates (amphibians and fish) and invertebrates (insects, nematodes and crustaceans) A natural host for WIV in New Zealand is the major pasture pest 'Porina' and the long-term goal of this research is to manipulate the virus for biological control purposes To achieve this an *in vitro* cell culture system was developed and time course studies carried out on WIV replication in the *Spodoptera frugiperda* cell line at permissive (22°C) and non-permissive (28°C) temperatures. Protein synthesis, mRNA transcription and DNA synthesis analysis showed that at the non-permissive temperature there was a decrease in all macromolecular synthesis except that of DNA Pulsed field gel electrophoresis of WIV-infected cells is being used to determine the nature of the DNA synthesized at both temperatures Using the replication strategy of Frog virus 3 (the analogous vertebrate Iridovirus) as a model for this group, it is postulated that at the non-permissive temperature of 28°C, replication of viral DNA is restricted to a primary phase only in which DNA is of genome length. At the permissive temperature of 22°C, secondary replication DNA intermediates are in the form of long concatemers. Comparative experiments are being conducted with bacteriophage lambda and T4 in order to establish the model of DNA intermediate formation. *(L)*

Spodoptera exigua and *Autographa californica* Multicapsid Nucleopolyhedroviruses (MNPV) Display DNA Replication Specificity

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The genome of the *Spodoptera exigua* MNPV was analyzed for the presence of putative origins of DNA replication. A set of cosmids and plasmids was tested in a transient replication assay Two origins (*Se*-hrl and a *Se*-non-hr) were identified. Detailed analysis of the *Se*-hrl sequence showed that the region contained four 60-bp-long palindromes with a central BglII restriction

site, located at mu 90 of the viral genome Hybridization of a single palindrome of *Se*-hrl with the SeMNPV genome revealed additional hr sequences (*Se*-hr2-5) located at mu 4, 30, 38 and 47. These four *Se*-hr's are being tested further for their ability to replicate. Both *Se*MNPV and *Ac*MNPV hr and non-hr origins were tested for their replication ability in the presence of either virus The hr origins could not be replicated by the heterologous virus, whereas the non-hr origins could This suggests a high degree of origin specificity in baculovirus DNA replication *(L)*

Expression of *p35*, the Apoptotic Suppressor Gene of AcNPV, in Non-permissive *Spodoptera littoralis* SL2 Cells

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Infection of *Spodoptera littoralis* larvae with the baculovirus *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcNPV) is abortive AcNPV infection of *S littoralis* SL2 cells yields low titers of viral progeny which correlate with induction of programmed cell death (apoptosis). Various studies showed that deletion mutants in the AcNPV *p35* gene (which encodes the apoptotic suppressor P35) induced apoptosis of permissive *Spodoptera frugiperda* cells. This suggested that the expression of the apoptotic suppressor *p35* is poor in AcNPV-infected SL2 cells and/or the synthesized P35 protein is not functional We detected low steady state levels of P35 in AcNPV-infected SL2 cells compared with those observed in *S. frugiperda* cells infected with AcNPV Moreover, expression of *p35* inhibited Actinomycin D-induced apoptosis of SL2 cells, indicating that the P35 protein is functional in this system. These data suggest that inefficient expression of functional P35 may determine the ability of AcNPV to replicate in SL2 cells Thus, overexpression of *p35* may enable the completion of the viral replication cycle through the suppression of apoptosis *(L)*

The *Spodoptera littoralis* Nuclear Polyhedrosis Virus (SlNPV) Encodes a Functional Ecdysteroid UDP-Glucosyltransferase

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The *Spodoptera* spp. are important lepidopteran pests which cause severe damages to major agricultural crops in many parts of the world The SlNPV member of the Baculoviridae, a pathogen of *Spodoptera* spp., is considered as a potential control agent of these pests Infection of *S littoralis* larvae with SlNPV revealed the phenomenon of delayed or blocked larval moulting. Such inhibition was attributed in other nuclear polyhedrosis viruses (NPVs) to the activity of a viral-encoded ecdysteroid UDP-glucosyltransferase (egt) In order to study further the interaction of SlNPV with its host, we isolated the *egt* gene from the viral genome using an *Autographa californica* NPV (AcNPV) gene-specific probe. Sequence determination of a 1925 bp portion of the hybridizing DNA fragment revealed an open-reading frame of 1548 bp that exhibits significant percentage of identity to egt identified in other NPVs The detection of egt activity in media of SlNPV-infected cells implied the production and secretion of a functional egt protein *(L)*

A Physical and Genetic Map of a 35 kb Region Containing the Polyhedrin Gene of the *Spodoptera littoralis* Nuclear Polyhedrosis Virus (SlNPV)

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The SlNPV pathogenic to the insect pest *Spodoptera littoralis* is considered as a potential control agent for *Spodoptera* spp. The future use of SlNPV for pest control requires genetic improvement of the virus-insecticidal properties Since little is known about the genetic organization of SlNPV, we aimed our research at the identification of potential sites in the viral genome to be used for genetic manipulations. Restriction enzyme analysis, Southern hybridizations and partial sequence analysis enabled us to establish a physical map for a region of ~35 kb containing the polyhedrin, protein kinase, p10, p74, ecdysteroid UDP-glucosyltransferase (egt) and ribonucleotide reductase genes The detailed characterization of SlNPV genes enables the design of a genetically engineered SlNPV The orientation of the SlNPV genes, special sequence features and comparison with other NPVs were discussed. *(L)*

Control of Lepidopteran Pests by Baculoviruses Expressing Novel Anti-insect Scorpion Neurotoxins

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Nuclear polyhedrosis viruses (Baculoviridae, group A) pathogenic to insects and not to mammals are appealing vectors for the control of lepidopteran insect pests Enhancement of the baculovirus rate of kill is required in order to meet the high standards of efficacy established by chemical insecticides. We have engineered a series of recombinant baculoviruses derived from the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV), which express toxins representative of the main groups of anti-insect neurotoxins from scorpion venoms (excitatory, depressant and α-toxin) Insect cells infected with the recombinant viruses secreted functional polypeptides. *Spodoptera littoralis* and *Heliothis armigera* larvae injected with budded viruses showed typical intoxication symptoms Bioassays performed on insect larvae fed with polyhedra showed that the recombinant viruses possessed improved insecticidal properties compared with wild-type AcNPV. *(L)*

Baculovirus-mediated Expression of Novel Anti-insect Scorpion Neurotoxins

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Recombinant baculoviruses expressing the LqhαIT and the AaIT anti-insect scorpion toxins isolated by us and others, were shown to possess enhanced insecticidal activity towards lepidopteran pests Pursuing this subject, we isolated new baculoviruses derived from the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) which express novel escitatory (LqhIT1) and depressant (LqhIT2) anti-insect selective toxins under the control of the *p10* and polyhedrin baculovirus promoters, respectively. *Trichoplusia ni* cells infected with the recombinant viruses secreted functional polypeptides Infection of *Spodoptera littoralis* and *Heliothis armigera* larvae with the recombinant viruses showed typical symptoms of contraction and flaccid paralysis characteristic of the excitatory and depressant toxins, respectively Bioassays performed on insect larvae fed with polyhedra showed that the recombinant viruses possessed enhanced speed of kill compared with wild-type AcNPV *(P)*

Sequencing and Characterization of a 5 kb Region of the *Helicoverpa zea* Single Nucleopolyhedrovirus North Carolina 1 (HzSNPV.NC1) Genome Containing the Polyhedrin Gene

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A baculovirus, HzSNPV.NC1, was isolated from the field Restriction endonuclease profiles of the virus showed the virus to consist of more than one genetically distinct isolate From the mixed population, one pure stock of virus was obtained, by plaque purification, for further study. *Eco* RI and *Sal* I genomic libraries of the viral DNA were produced, and screened, to identify clones containing the polyhedrin gene and flanking regions. The polyhedrin gene and flanking regions have been sequenced and characterized. Within this region of the HzSNPV.NC1 genome, an open-reading frame (ORF) showing homology to ORF 23 (previously called ORF 9) of *Autographa californica* NPV, has been identified. The function of this ORF is unknown. Gene knock-out analysis is being used to help determine the function of this ORF, and to determine whether the ORF is essential or non-essential. *(P)*

Comparative Analysis of Two Baculoviruses Infecting *Helicoverpa armigera*

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Helicoverpa (=Heliothis) armigera is an economic pest of various important crops in southern Africa It is reported to have developed resistance to commonly used chemical insecticides in some parts of the world such as Australia and Thailand. Alternative control strategies based on microbial control are being sought worldwide. Two baculoviruses have been found infecting field populations of *H armigera*; they could form important alternative control strategies Prior to biological studies to determine the virulence and efficacy of the two viruses, comparative biochemical studies are being carried out. Studies of the structural proteins by SDS-PAGE have shown that the matrix proteins of both viruses have the same size, as do most of the other structural polypeptides from the viral components. Electron microscope studies have confirmed that one of the viruses is a nuclear polyhedrosis virus while the other is a granulosis virus. Restriction enzyme studies have shown different profiles for the two viruses. Physical restriction maps are being constructed *(P)*

Development of Immediate-early Recombinant Baculovirus Pesticides

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Recombinant viruses that express insect-specific toxins under the control of viral late gene promoters kill susceptible lepidopteran hosts faster than wild-type viruses. In order to improve further the efficacy of genetically engineered baculovirus pesticides, we produced a recombinant that contains an insect-specific toxin gene (AaIT) under the control of an immediate early promoter (*ie1*) of *Autographa californica* nuclear polyhedrosis virus In cultured cells infected with the recombinant, AaIT protein was detected as early as 4 h post-infection. AaIT accumulated as the infection progressed and, even as late as 24 h post-infection, the amount of AaIT was equal to that produced by a recombinant that expressed AaIT under the control of the very late *p10* promoter. Similar results were obtained with a recombinant that expressed a modified insect juvenile hormone esterase (JHE-KK) under *ie1* control, confirming the ability of this promoter to provide early and abundant expression of proteins with potent pesticidal activity. Bioassays showed that the *ie1*-AaIT recombinant killed *Heliothis virescens* larvae faster than wild-type virus Larvae infected with the *ie1*-AaIT recombinant were smaller than those infected with the *p10*-AaIT virus, suggesting that expression of AaIT earlier in infection enhanced the ability of the virus to reduce the feeding activity of *H. virescens* larvae *(L)*

Insect Small RNA Viruses Offer New Strategies for Pest Control

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Small non-enveloped RNA viruses of insects, such as the tetraviruses, have been little used as pest control agents. However, modern techniques of genetic engineering allow the unique properties of these viruses to be exploited for new approaches to pest control. One example involves virus production in non-host systems such as in transgenic plants *Helicoverpa armigera* stunt virus (HaSV) is a recently characterized tetravirus with significant potential for the control of the cotton bollworm. This virus is specific for host midgut cells, rapidly inducing a massive increase in shedding of rejected midgut cells in neonate larvae to the extent that the regenerative cells are unable to maintain a functional midgut, and the larvae become stunted and die. HaSV-induced apoptosis appears to underlie this cell-shedding, which may be considered a primitive immune response. The HaSV genome comprises two RNA strands, carrying only three genes. We have constructed synthetic genes designed to express the virus genome and produce infectious virus particles in non-hosts such as plants or insect cells infected using a baculovirus vector. Initial data from transgenic plants indicate that infectious virions are assembled in the plants and can protect them against feeding damage. *(L)*

Tetravirus Molecular Biology and Evolution

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The tetraviruses are small (40 nm) non-enveloped RNA viruses with T=4 icosahedral surface symmetry which infect only species of Lepidoptera. These unique insect viruses may offer significant insights into the evolution of RNA viruses. The family has recently been divided into two genera, one containing viruses like the type member *Nudaurelia* β virus (NβV), with a single, non-polyadenylated RNA of 6 kb; the other containing two viruses – *Nudaurelia* ω virus (NωV) and *Helicoverpa armigera* stunt virus (HaSV), with bipartite genomes A surprising feature of the HaSV RNAs is the presence of an aminoacylatable 3'-

$tRNA^{Val}$-like structure, which lacks a pseudoknot and is the first found on an animal virus. HaSV RNA 1 encodes the 187 kDa RNA replicase, which shows homology to those of viruses in the alpha-like superfamily, and RNA 2 encodes the 71 kDa capsid precursor and an overlapping 17 kDa protein Sequence analysis of the NβV genomic RNA shows it to encode the putative replicase (140 kDa) at the 5' end, followed by the capsid precursor gene (70 kDa). NβV and HaSV are only distantly related, with homologies of 33% for the polymerase and 24% for the capsid protein. We propose that the bipartite genome evolved from the monopartite form Preliminary evidence suggests that the p17 protein found on HaSV but not on NβV may have evolved *de novo* to regulate replication of the two genomic RNA components *(L)*

Identification of Baculovirus iap Genes

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Baculovirus-encoded inhibitors of apoptosis, *e.g. Autographa californica* nucleopolyhedrosis virus (AcMNPV) *p35*, have been implicated in extending virus host range *in vitro* and *in vivo*. To date, *p35* has been identified only in AcMNPV Functionally related genes, such as *Cydia pomonella* granulovirus (CpGV) and *Orgyia pseudosugata* NPV *iaps*, may have a similar role. In an attempt to identify other baculovirus genes related to *p35* or *iaps*, we have used an assay system developed by Crook *et al* (1993) to isolate these sequences. *Spodoptera frugiperda* cells were co-transfected with DNA from an AcMNPV mutant deficient in *p35* and intact genomic DNAs from ten baculoviruses, including NPVs and GVs A cloned copy of the CpGV *iap* was used as a positive control Preliminary results suggest that two virus DNAs, *Mamestra brassicae* MNPV and *Spodoptera littoralis* MNPV, were able to rescue the AcMNPV *p35* mutant and facilitate normal virus replication in *S. frugiperda* cells. These results extend the number of baculovirus isolates which are now known to harbor functional *p35* or *iap* genes. *(P)*

Identification of the *Mamestra brassicae* Nuclear Polyhedrosis Virus (MbMNPV) *gp37* and *PTPase* Genes

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A 1 8 kbp region upstream of the MbMNPV *egt* gene has been sequenced and found to include one partial and two complete open-reading frames (ORFs). These show similarity to the *Autographa californica* NPV *ORF13*, *gp37* and *PTPase* genes, respectively The *gp37* ORF is also similar to other baculovirus *gp37* genes and to entomopoxvirus fusolin genes Interestingly, the *PTPase* gene shows only limited similarity to AcMNPV *PTPase* It is more similar to various mammalian *PTPases*, suggesting that AcMNPV and MbMNPV acquired their *PTPase* genes independently MbMNPV and AcMNPV show very different gene arrangements in this region *ORF13* and *egt* are in similar relative positions in both viruses, but the orientation of *ORF13* is reversed in MbMNPV In AcMNPV, the *lef1* gene is found between *ORF13* and *egt*. However, the *PTPase* gene occurs 10 kbp upstream of *egt*, and *gp37* is found 40 kbp downstream. MbMNPV *gp37* and *PTPase* have been subcloned, expressed in *Escherichia coli*, and purified The preparation of antisera to the purified proteins and the assay of PTPase activity are in progress *(P)*

The *Mamestra brassicae egt* Gene: Phylogenetic Analysis and Gene Disruption

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The *egt* gene of the *Mamestra brassicae* nuclear polyhedrosis virus (MbMNPV) has been cloned and characterized Phylogenetic trees of NPVs have been generated based on the alignment of baculovirus EGT sequences. These suggest that MbMNPV is a group II NPV. Comparison of the EGT-based phylogenies with those for polyhedrin/granulin suggest that AcMNPCV acquired its polyhedrin gene by recombination. The *egt* gene of MbMNPV is being disrupted in two ways First, site-specific mutagenesis has been used to delete a *Kpn*I restriction site present within the coding region of the gene. This results in disruption of the *egt* reading frame and loss of EGT activity as evidenced by transient expression assays of insect cells co-transfected with this construct and an *egt*-AcMNPV virus. Co-transfection of this construct with MbMNPV will allow screening for an *egt*-virus The second approach utilizes the yeast *Saccharomyces cerevisiae*. DNA sequences sufficient for maintenance and selection in *S cerevisiae* have been inserted into the cloned *egt* gene. Co-transformation of *S. cerevisiae* with this construct and MbMNPV viral DNA will allow selection of viral clones disrupted in *egt*. These viruses will facilitate further laboratory analysis and field studies investigating the role of *egt* in viral fitness. *(L)*

Production of Potato Leafroll (PLRV) Virus-like Particles (VLP) in Insect Cells Infected with Recombinant Baculoviruses

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A full-length cDNA copy of PLRV RNA was constructed in the transfer vector pVL1392 and recombined with AcNPV DNA to give a recombinant baculovirus (pSAB55). *Spodoptera frugiperda* or *Mamestra brassicae* cells were infected with pSAB55 and examined for the expression of PLRV genes. Proteins encoded by both 5' and 3' parts of the PLRV genome were detected by immunoblotting Sections of infected cells showed that VLP accumulated in the nuclei, often in crystal-like masses Buffer extracts contained VLP which were indistinguishable from PLRV particles by electron microscopy or sedimentation behavior. Although the RNA in the VLP contained PLRV sequences, no genome-sized molecules could be found. It was shown previously that the coat protein gene assembled in this system only when modified with a histidine tag Expression of more of the PLRV genome appears to mimic the effect of the histidine tag. *(P)*

Sequence Expression and Promoter Activity of a Novel Gene of *Leucania seperata* Nuclear Polyhedrosis Virus (LsNPV)

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A 819 bp Sal I/Hind III fragment of LsNPV DNA was sequenced Analysis using DNASIS software revealed that this fragment contained an open-reading frame (ORF) spanning from 305 to 646 bp which encoded a polypeptide of 114 amino acid

residues In the 5' flanking region of the ORF, the TATA box, CAAT box, early gene regulatory elements ACGT and GC motifs and transcriptional initiative consensus sequence TTAAG of late gene were present A mini ORF coding 11 amino acids was also mapped in this region. 3 bp upstream, stop codon TGA presented a AATAAA poly A signal, and downstream TGA existed a double stem-loop structure It was possibly a novel gene and named p13 gene because of no homologies in nucleotide sequence and amino acid residues sequences between the ORF and other published baculovirus genes A leucine zipper structure (Thr-6aa-[Leu-6aa]$_3$) and two leucine zipper-like structures (Leu-7aa-Thr-7aa-[Leu-7aa]$_2$ and [Leu-6aa-Val-6aa-Tyr]$_2$-6aa-Cys) were found in the C- and N-termini of the predicted protein, respectively, which were designated as leucine trans-conformation and LVT repeat structures. With prokaryotic expression vector pBV220, p13 protein was expressed in *Escherichia coli* strain TG1, and the MW of the product calculated by SDS-PAGE was ~14 kDa, similar to that of deduced protein A reporter gene, the green fluorescent protein (GFP) gene, was chosen to be fused with the SalI/SmaI and SalI/ClaI fragments, respectively, which encompass the positions -305 – -29 and -305 – -3 of the p13 promoter to place the GFP gene ORF under the control of the p13 promoter, and two constructs: pLsp13-2G and pLsp13-3G, were derived. The activity of the p13 promoter was assayed by measuring the fluorescence in intact Ls cells transfected with the plasmid constructs by the calcium phosphate co-precipitation method. The p13 promoter was active when co-transfected with cells with plasmid construct and LsNPV DNA, but not active when transfected alone with plasmid construct This provided evidence that the p13 gene was a delayed-early gene *(L)*

Complete Sequence of the BamHI J Fragment of *Cydia pomonella* Granulosis Virus (CpGV)

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The 5 2 kbp BamHI J fragment of CpGV has been subcloned, subjected to saturating transposon mutagenesis in *Escherichia coli*, and sequenced in its entirety. Fifteen putative open-reading frames (ORFs) of at least 50 amino acids have been identified in this sequence, only five of which show similarity to known proteins. Two of these (IAP and the 41 kDa ODVP-6E homolog) have been described previously. Of the other three, one is homologous to *Autographa californica* NPV (AcMNPV) ORF29, an 8 6 kDa ORF that is close to AcMNPV IAP1 A partial ORF is highly similar to a *Lymantria dispar* NPV (LdMNPV) ORF (ORF6) of unknown function that is located close to the LdMNPV calyx gene. Finally, a partial ORF shows some similarity to sigma-1 proteins of mammalian reoviruses and to baculovirus p10 proteins Close examination of the latter homology reveals that it is in regions of these proteins believed to form coiled coil structures and thought to be involved in aggregation. *(P)*

Filament Webs Spun by *Amsacta moorei* Entomopoxvirus

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Entomopoxviruses are insect viruses which produce occlusion bodies or spheroids in the cytoplasm of the infected cell. These structures are composed primarily of a cysteine-rich protein called spheroidin. We identified another viral gene product in insect cells infected with *Amsacta moorei* entomopoxvirus which is expressed at high levels late in infection. This protein migrates as a 25/27 kDa phosphoprotein doublet on SDS polyacrylamide gels, and we have named it FALPE (filament associated late protein of entomopoxviruses) The gene for this protein was sequenced and FALPE was found to contain an extensive stretch of proline and glutamic acid residues Electron microscopy and immunofluorescence studies revealed that this protein formed cytoplasmic filaments in the infected cell which were associated with the occlusion bodies FALPE may play a role in viral and/or occlusion body morphogenesis of this entomopoxvirus. *(L)*

Filovirus-like Particles Detected in Extracts from the Leafhopper *Psammotettix alienus*

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Filamentous particles were detected by negative contrast electron microscopy (0.5% PTA or 2% uranyl acetate) of extracts from the leafhopper *Psammotettix alienus* reared on healthy *Festuca gigantea* plants The particles were straight, slightly curved or flexuous, often with one end curled like a walking stick or curled into a ring with an outer diameter of ~200 nm. Some particles seemed broken, but still interconnected Projections, 8–10 nm long and ~10 nm apart, were evenly distributed on the surface. The diameter of particles, including projections, was 55–70 nm. Length distribution of 280 particles showed two size classes: 450–750 nm (26% of particles, median length ~600 nm) and 900–1400 nm (63% particles, median length ~1100 nm). The longest measured particle was ~2400 nm. Many particles had one end rounded and the other truncated At the truncated end, a central canal with a diameter of 5–10 nm may be seen. Most particles did not reveal any internal structure, but when kept in stain for 6 days at 5°C, partly disintegrated particles showed internal cross striation with a periodicity of 5–5.5 nm. The diameter of this internal part was ~30 nm. Morphologically, the particles detected are similar to virions of Marburg and Ebola viruses belonging to the virus family Filoviridae, but can be distinguished by having a smaller diameter (55–70 nm compared with ~80 nm for Filoviruses) No Filovirus-like particles were detected in extracts from the plants used for rearing the leafhoppers. *(P)*

Comparative Studies of Properties of Insect DNA Viruses and the Penaeid Rod-shaped DNA Virus, Newly Isolated from Penaeid Shrimp

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Mass mortalities caused by a new viral disease of kuruma shrimp, *Penaeus japonicus*, occurred in 1993 Electron microscopic observations revealed that the virions were ovoid-shaped, and contained a partially lenticular-shaped cylindrical nucleocapsid measuring 84 nm × 226 nm within the loosely surrounding envelope No occlusion bodies were found in the cells infected with the virus. Nucleic acids from the purified virus particles consisted of a non-segmented, double-stranded DNA molecule with a size comparable to the DNA genome of *Oryctes* virus The viral DNA did not hybridize with the DNA genome of a baculovirus, the *Bombyx mori* nuclear polyhedrosis virus. Sequence data analysis based on the partial DNA sequences of viral genome failed to identify any closely related DNA viruses in the databases These results suggest that the virus is one of the unassigned rod-shaped double-stranded DNA viruses of invertebrates such as *Oryctes* virus, which were previously classified in the Nudibaculovirinae, a subfamily of the Baculoviridae. Therefore, the virus was designated penaeid rod-shaped DNA virus *(L)*

Poly (Tri or Bi)-Cistronic Phytoreoviral Segment Translatable in Both Plant and Insect Cells

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Sequence analysis of genomic segment S12 of rice dwarf virus (RDV) and segment S9 of wound tumor virus (WTV), both members of the genus *Phytoreovirus*, previously identified small out-of-phase overlapping reading frames (ORFs) To determine whether these small ORFs are expressed *in vivo*, antibodies were generated against protein products of the large and small ORFs produced with a baculovirus vector in insect cells and in an *Escherichia coli* expression vector system, respectively. Western analysis revealed that RDV S12 mRNA specified translation products from the large and two overlapping small ORFs (Pns12; 312 aa, Pns12OPa, 92 aa and Pns12OPb, 84 aa, respectively) in both rice plant hosts and in *Spodoptera frugiperda* insect cells. Similarly, WTV S9 mRNA was found to direct the synthesis of protein products from both the large and small out-of-frame ORFs in *S frugiperda* cells Results of site-specific and deletion mutagenesis studies were consistent with a *leaky scanning* translation mechanism for the synthesis of the small ORFs These results provide the first example of a tricistronic mRNA for a segmented double-stranded RNA virus The conserved nature of the mechanism of *in vivo* expression and the deduced amino acid sequence for the phytoreovirus out-of-frame small ORFs suggest a functional role for the protein products during the viral replication cycle. *(P)*

Evidence of Independent *Leaky Scanning* as the Mechanism of Translation Initiation in Plum Pox Virus (PPV)

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PPV is a member of the *Potyvirus* genus, the largest group of plant-infecting viruses The genome of PPV is a messenger-polarity RNA molecule 9786 nt in length, with a VPg protein at its 5' end and a poly(A) tail at the 3' end It contains a single open-reading frame (ORF) that is translated into a large polyprotein which is co- and post-translationally processed into the functional proteins. Although the ORF starts at nucleotide 36 ($_{36}$AUG), it is translated from the second $_{147}$AUG, which is in a more favorable initiation context The presence of an internal ribosome entry site in the 5' ncr has been proposed for two members of this genus. Nevertheless, we have carried out *in vitro* translation and transient expression analysis in protoplasts of a nested set of short deletion mutants and the results show that no sequence in the 5' ncr of PPV is necessary for efficient translation initiation; translation assays of long deletion mutants have shown that no secondary structure is necessary and also denied the existence of redundant sequences that become active when others are deleted On the other hand, when the cryptic $_{36}$AUG was placed in a favorable context for translation initiation, it turned into an efficient initiation codon *in vitro* Furthermore, *in vivo* experiments have shown that transcripts synthesized from cDNA clones with the nucleotides 73 to 145 deleted were able to infect plants. These results point strongly to *leaky scanning* as the mechanism of translation initiation in PPV – but a peculiar *leaky scanning*, where the initiation of translation does not require a cap structure at the 5' end, as shown by transient expression experiments using methylated and unmethylated capped RNAs. The independence of a cap structure at the 5' end of the mRNA is congruent with the absence of a complex secondary structure at the 5' ncr. The unstructured nature of the PPV RNA leader, predicted by computer programs and by chemical and enzymatic probing, might make unnecessary the binding of a factor with helicase activity (eIF-4F complex) that enables the ribosomes to scan the leader towards the initiation codon *(L)*

A Sequence in the 3' Untranslated Region of Barley Yellow Dwarf Virus (BYDV)-PAV RNA is Required for Cap-independent Translation

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Translation of the 5'-proximal (39K) ORF of BYDV-PAV genomic RNA is initiated efficiently in the absence of a 5' cap only in the presence of a 100 base sequence (called the 3' translation enhancer, 3'TE) located 5 kilobases downstream Deletion of this sequence reduces translation of uncapped mRNA by over 30-fold. Capping the mRNA restores translation efficiency. By all tests, *in vitro* and *in vivo*, the 3'TE (in conjunction with the 5'UTR) functionally substitutes for a 5' cap but not a poly(A) tail. This differs from other cap-independent translation elements (*e.g.* IRES) which are located entirely 5' of the start codon. *Trans*-acting factor(s) are implicated in the communication between the 3'UTR and the AUG at the 5' end Acting *in trans*, the 3'TE inhibits translation of both uncapped mRNA containing the 3'TE and capped mRNA lacking the 3'TE Addition of eukaryotic initiation factor complex eIF-4F stimulated translation of uncapped mRNA lacking the 3'TE, but had no effect on translation of 3'TE-containing or capped mRNA in wheat germ extracts. The *trans*-inhibition of translation by the 3'TE can be reversed by exogenous eIF-4F Thus, the 3'TE mimics a 5' cap in its apparent ability to recruit eIF-4F and to stimulate translation initiation. The 3'TE is required for virus replication, and a conserved sequence within the 3'TE was discovered in diantho- and necroviruses. *(L)*

Ribosomal Shunt during Translation of Rice Tungro Bacilliform Virus RNA

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The expression of the rice tungro bacilliform virus open-reading frame I was studied in transiently transfected protoplasts. Expression occurs despite the presence of a long leader sequence and the absence of a proper AUG initiation codon. Translation is initiated at an AUU codon. The efficiency of initiation in rice protoplasts depends strongly on the mechanism by which ribosomes reach this codon. From the effects of scanning-inhibiting structures inserted into different leader regions, it may be deduced that this mechanism is related to the ribosome shunt described for cauliflower mosaic virus 35S RNA The process delivers initiation competent ribosomes to the region downstream of the leader and is so precise that only the second of two potential start codons 12 nucleotides apart is recognized The AUU codon that is used when present downstream of the leader is hardly recognized as a

start codon by ribosomes that reach it by scanning. *(L)*

Expression of the Coat Protein of Potato Virus X (PVX) from a Dicistronic Messenger RNA in Transgenic Potato Plants

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Transgenic potato plants were generated which express a dicistronic mRNA corresponding to the 8K and coat protein (CP) genes of PVX. Northern blot analysis of total and polysomal RNA isolated from these plants has indicated that the 8K and CPs are translated from a single, functionally active dicistronic mRNA Expression of both of these proteins in transgenic plants was demonstrated by Western blot analysis, and suggests that translation of the CP takes place either by internal initiation or by a termination/reinitiation mechanism When a hairpin structure is placed in front of the 8K gene, expression of this protein is drastically reduced. However, the expression of the CP is not affected These results demonstrate that the expression *in planta* of the CP gene takes place by an internal initiation. The expression of the CP of potexviruses may take place from the polycistronic genomic as well as from the amplified CP subgenomic RNAs. *(P)*

Expression of Genes Located on the Subgenomic RNA of Potato Leafroll Virus (PLRV)

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The genome of PLRV consists of a single positive sense RNA, ~6000 nt in length. The analysis of the nucleotide sequence has revealed the presence of six open-reading frames (ORFs). Three ORFs (ORF1, ORF2 and ORF3) are translated from the genomic RNA, whereas the other three (ORF4, ORF5 and ORF6) are translated from a subgenomic RNA (sgRNA). A better knowledge of the mechanism of PLRV genome expression would be useful for efficient protection of potato plants against viral infection. Therefore, studies were undertaken of the expression of ORFs located on the sgRNA. Expression of the ORF4 and ORF5 was examined using *in vitro* systems, the results obtained indicate the following (i) the ORF4 and ORF5 encoding for coat protein and 17K, respectively, are translated from the same RNA template by initiation at out-of-frame AUG initiation codons, (ii) translation of the 17K protein can be initiated on either the first or the second AUG codon in ORF5, even though the second AUG codon is in a better context; (iii) suppressor tRNAs for AUG stop codon significantly increase efficiency of translation of ORF6; and (iv) the presence of the subgenomic leader sequence significantly decreases expression of both ORF4 and ORF5 *(P)*

Effect of the Novel Small RNA Detected in Wheat Embryo Ribosomes on Translation of mRNA with Viral 5'-Leader Sequence

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The influence of heat shock (HS)-inducible 5 3S RNA on the *in vitro* polypeptide synthesis at normal and HS temperatures was studied Two mRNAs, each coding for GUS and differing only in the structure of the 5'-untranslated region (5'-UTR), were used as messengers. One of them contained (whereas the other did not) the 5'-UTR from TMV RNA (omega), which is known to confer to the non-HS mRNAs the ability to be translated efficiently under HS conditions. The omega is also known to have the translation enhancer activity. Direct addition of purified 5.3S RNA to WG cell-free translation system resulted in increase of the omega-dependent enhancing effect from 6- to 12-fold at normal (26° C) temperature and from 10- to 350-fold at HS (37° C) temperature. Analogous addition of 5S or 5.8S ribosomal RNAs did not result in similar changes. These data demonstrate that 5.3S RNA has a clear discriminatory effect on the translation of different mRNAs and the mechanism of this effect requires an additional detailed investigation *(P)*

XXV: STRUCTURE AND REPLICATION OF PLANT VIRUSES

Structure and Function of the Banana Bunchy Top Virus (BBTV) Genome

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BBTV is a circular ssDNA virus with isometric virions ~20 nm in diameter. Its genome consists of at least six ssDNA components (BBTV components 1 to 6) ranging in size from 1018 to 1111 nt Each of these components has been identified in all BBTV isolates tested and each contains one large open-reading frame (ORF) in the virion sense The component 1 major ORF putatively encodes a replicase-associated protein (RAP) and interestingly there is a second functional ORF internal to this major ORF. The major ORF of component 3 encodes the viral coat protein. The function of the four other components is not yet known The intergenic region of each component contains a conserved stem-loop sequence, a conserved major common region (with an associated primer) and has promoter activity. We and others have isolated additional components that potentially encode RAPs. The organization of these additional RAP-encoding components is different from that of BBTV components 1–6 and we have demonstrated that they are not present in all isolates and therefore may not be essential components of the BBTV genome. There are four other viruses with similar characteristics and, along with BBTV, probably represent a new group of plant viruses These viruses also appear to be different from the Circoviruses, small isometric ssDNA viruses that infect animals. *(L)*

RNA Transcripts of Banana Bunchy Top Virus (BBTV)

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We have mapped the RNA transcripts of BBTV components 1–6. Northern hybridization studies and 3' RACE have identified seven polyadenylated RNAs associated with the BBTV genome Interestingly, component 1, which contains one major open-reading frame (ORF), a putative replicase-associated protein (RAP), has been shown to transcribe a mRNA from an ORF internal to the major RAP ORF. This transcript encodes a putative M, 5K protein of unknown function It is possible this internal ORF may be involved with regulation of viral replication. Both component 1 RNA transcripts have been identified in tobacco plants transformed with a vector containing the major ORF of component 1. Component 2 was found to have an RNA transcript which mapped to a small ORF This is the first report of an ORF encoding a putative protein from component 2. The RNA transcripts associated with components 3–6 are all from previously reported ORFs. We have mapped the important 3' untranslated regions, including the conserved termination signals. The poly(A) signal, poly(A) site, GT-rich region and an upstream element (A/C/TTGTAA) seem to be also conserved in other ssDNA viruses. *(L)*